Postmitotic Reconstruction of Nucleoli in Culture Cells with UV-Microbeam Photoinactivated Centrosome

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Ultraviolet microirradiation of one of the poles of the mitotic spindle of PK cells was performed 1 min after the onset of the anaphase. Formation of the nucleolus in the telophase and G_1 period was studied by vital observation, electron microscopy and indirect immunofluorescence using antibodies against B23 protein. Sister cells with nonirradiated centrosomes and cells with partially irradiated cytoplasm were used as controls.

During the first hour after the anaphase, the nuclei in both sister cells were identical and contained numerous small dense particles with granular ultrastructure. B23 protein detected in the mitotic poles and at the chromosome surface in the anaphase was dispersed in the cytoplasm in both cells in the early G_1 period. Later, control cells did not display any difference from intact cells: nucleoli of a typical structure were formed, B23 protein appeared in the karyoplasm and was then accumulated in the nucleoli and disappeared from the cytoplasm and karyoplasm. Nucleoli in cells with irradiated centrosomes did not achieve the normal size and contained a significantly lower amount of granular component. B23 protein was dispersed in the karyoplasm and was not accumulated in the nucleoli. Nucleoli in cells with irradiated centrosomes contained small dense particles for at least 24 h.

Telophase cells where microtubule formation had been inhibited by nocodazole formed normal nucleoli. It shows that the effects observed in cells with irradiated centrosomes are not due to the absence of the microtubule radial system. We conclude that UV microirradiation of the mitotic centrosome disturbs the postmitotic reconstruction of nucleoli probably because of the photodestraction of B23 protein accumulated in the mitotic pole.

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In previous works [1-4] we have shown that after irradiation of the centrosome in the early anaphase the cell completes mitosis, but in the daughter cell the irradiated centrosome is not the centre of microtubule formation. Cells with irradiated centrosome survive in culture for a long time, but differ from sister and intact cells. In particular, cells containing irradiated centrosome are not capable of forming normal nucleoli [1]. The physiological parameters of such cells differ from those in sister cells, in particular, the level of RNA synthesis in them is decreased [4].

This paper studied the formation of nucleoli and the distribution dynamics of B23 protein in PK cells after UV microirradiation of one of the poles of the mitotic spindle in the anaphase.

B23 protein is widespread in cells of higher eukaryotes, its molecular weight is 37-38 kDa and pI - 5.1-5.2 [5, 6]. The major function of B23 is, apparently, associated with the assembly of ribosomal particles [6-8]. This protein has been found to be constantly exchanged between the nucleus and the cytoplasm and be able to be coupled with the nuclear signal sequences of other proteins, for instance, Rex and Rev viral proteins [9-11]. This is an indirect indication that B23 is possibly involved in nuclear-cytoplasmic exchange.

Mitotic division is accompanied with reversible inactivation of the ribosomal genes and breakdown of the nucleolus. This leads to the redistribution of most nucleolar proteins [12]. Part of the nucleolar proteins get to the chromosomal matrix and/or are distributed in the cytoplasm of the cell [13]. As a rule, such proteins as B23, which in the interphase are located in the granular part of the nucleolus, enter the cytoplasm [14, 15]. A recent study has shown that B23 is in the spindle poles during the mitosis [16].

In the telophase, the ribosomal genes are activated and the structure of the nucleolus is restored [12, 17]. This is preceded by the formation of a large number of small intranuclear particles, containing a number of proteins of the mature nucleoli. This process can be blocked by actinomycin D or micro-injection of antibodies against RNA polymerase I [12].

In this paper, we show that microirradiation of a pole of the mitotic spindle by UV light in the anaphase leads to disturbances in the postmitotic reconstruction of the nucleoli and prevents the accumulation of B 23 protein in them.

EXPERIMENTAL

Microirradiation and vital observations. Pig kidney (PK) cells were used as an object of study. The cells were grown on round quartz coverslips, which then were mounted into an original chamber for vital observations [3]. Medium 199 with 10% fetal cattle serum supplemented with 100 µg/ml

gentamicin was used for culturing.

A special setup described in detail earlier [3, 18] was used for microirradiation. Microirradiation was performed via a band-pass filter ($I_{max} = 280$ nm) using an Ultrafluar 100/1.25 quartz lens (Opton, Germany) (glycerol immersion). The diameter of the UV-light beam in the focal plane of the objective lens was about 1.6 µm. The irradiation power density was 4-10⁻² erg/(µm²-s). The vital observations of the cells were carried out in phase contrast (objective lens Plan-Neofluar 40/0.9 (Opton, Germany), glycerol immersion). The cells were photographed using an MFN-12 camera attachment on Mikrat-300 film (Tasma, Russia).

Immunocytochemistry. For immunocytochemical staining, the cells were washed in phosphate buffered saline (PBS), fixed for 20 min with 3% formaldehyde in PBS and lyzed for 1 min in 1% solution of Triton X-10 in PBS at room temperature. The preparation was washed in PBS and stained with murine antibodies against B 23 and then with antimurine antibodies conjugated with Texas Red. Fluorescence of Texas Red-labelled antibodies was observed at a wavelength of more than 520 nm at an Opton-3 photomicroscope (Germany) and photographed on RF-3 film (Tasma, Russia). Simultaneously, the same cells were photographed in phase contrast (Mikrat-300 film, Tasma).

Electron microscopy. For electron microscopy, the cells were fixed in 2.5% glutaraldehyde on Sorenson's buffer (pH 7.2-7.4), then fixed with 1% OsO₄, dehydrated and embedded into a mixture of Epon-812 according to the standard technique. Serial ultrathin sections of irradiated cells were prepared on an LKB-3 ultratome (Sweden), examined and photographed on HU-11B and HU-12 electron microscopes (Hitachi, Japan) at an accelerating voltage of75kV.

RESULTS

One of the poles of the mitotic spindle of the PK cells was subjected to UV microirradiation (280 nm) for 15 s in the early anaphase (Fig. *1a*). After irradiation of the pole the cells were capable of completing the mitosis. Cyto-kinesis in such cells ended 15-20 min later than in intact cells.

In 40-60 min after irradiation the nucleoli of irradiated and sister cells looked typical of this culture at this stage of the cell cycle. Many small dense particles, among which it was impossible to differentiate the true nucleoli at the light-microscopic level, were formed in the nuclei of the cells with the irradiated centrosome and in the nuclei of the sister cells (Fig. *1b*).

In 60 min after irradiation, chromatin in the nuclei of the irradiated and sister cells was almost totally decondensed. The nucleoli in the nuclei of the sister cells had a typical nucleolonemic ultrastructure, the developed granular



Figure 1. PK cells after UV microirradiation of a pole of a mitotic spindle at anaphase. Vital observations, phase contrast, *a*, The mother cell at anaphase 1 min after irradiation of the centrosome; *b-d*, daughter sister cells in 30 min, 1 h 50 min and 6 h 15 min, respectively, after irradiation of the centrosome of the mother cell; I, a cell with the irradiated centrosome; N, a sister cell with the non-irradiated centrosome; the large arrow shows the irradiated pole; small arrows point to the prenucleolar bodies. Bar, 10 μ m.

and dense fibrillar components and small fibrillar centres. By their structure and size, they did not differ from the nucleoli of the normal interphase cells in the early G_I -period (Fig. *Id*). In the nuclei of the cells with the irradiated



Figure 2. The ultrastructure of the nucleoli of the cells after irradiation of the centrosome at mitosis, *a*, *b*, *d*, a sister cell with the non-irradiated centrosome; *c*, *e*, a cell with the irradiated centrosome; a-c, sister cells 64 min after irradiation; *d*, *e*, sister cells 4.5 h after irradiation; DFC, the dense fibrillar component; FC, the fibrillar centre; Gr, granules (the arrows show the prenucleolar bodies). Bar, 1 µm.

centrosome, the nucleoli sharply differed by their ultrastructure from the nucleoli of the sister cells. In these cells, the nucleoli contained single fibrillar



Figure 3. Immunocytochemical staining of cells by antibodies against B23 protein after irradiation of the centrosome at mitosis, *a*, *b*, sister cells 3.5 h after irradiation; *c*, *d*, sister cells 7.5 h after irradiation; *a*, *c*, immuno-fluorescence; *b*, *d*, phase contrast of the same cells; I, a cell with the irradiated centrosome; N, a sister cell with the non-irradiated centrosome; the arrows show the nucleoli. Bar, 10 μ m.

centres, had less granules and were distinctly segregated into zones corresponding to the fibrillar centres, the dense fibrillar component and the granules (Fig. 2c).

Small dense particles observed on the light-microscopic level both in irradiated and sister cells looked the same in both groups of cells on the electronmicroscopic level and appeared as dense aggregates of granular material. They were formed by granules about 15 nm in diameter and fine fibrillai material (Fig. 2b and c, arrows). Such particles, usually called prenucleolai bodies, are normally present in the karyoplasm of the cells in the early G_1 period and contain granules of RNP [19].

At the early times after irradiation (40-60 min) the distribution of B23 protein in the cells containing the irradiated centrosome and in their sistei cells did not differ from the distribution of this protein in intact cells in the early G_1 period. B23 protein was diffusely distributed in the cytoplasm and was also located in the cytoplasm as large and small clusters; in the nuclei oi the cells the protein was totally absent (data not shown).

Later, in 2-4 h after irradiation, the number of small dense particles in the nuclei of sister cells decreased to one or two and the true nucleoli were formed, which had a pronounced nucleolonemic structure. No nucleoli with the normal structure were formed in the nuclei of the cells which received the irradiated centrosome. Among the multitude of dense particles, several larger particles typical of the given culture stood out in the nuclei of such cells (Fig. 1c).

At these times, after irradiation the distribution of B23 protein in sister cells to irradiated cells did not differ from the distribution of this protein in intact cells: we observed a bright immunochemical staining of the nucleoli, whereas in the karyoplasm and cytoplasm the staining was absent (Fig. *3a*). The localization of B23 protein in cells with the irradiated centrosome sharply differed from that described above: in the nuclei of such cells the nucleoli were stained significantly weaker than in sister cells; besides, the karyoplasm was stained rather intensively (Fig. 3a).

Significant differences between the nuclei of the irradiated and sister cells were manifest 4-8 h after irradiation. Small dense particles in the nuclei of the sister cells totally disappeared, the nucleoli formed reached their usual size (Fig. *4a*). The nucleoli contained numerous granules and small fibrillar centres covered by a solid layer of the dense fibrillar component (Fig. *2d*). The cells containing the irradiated centrosome formed no normal nucleoli and, as in the early G₁ period, contained small dense particles of granular structure (Figs. 1 and *4b*). The nucleoli in the nuclei of these cells were virtually devoid of granules, contained one large fibrillar centre shifted to the periphery, and the overdeveloped dense fibrillar component (Fig. *2e*).

At the same time, the karyoplasm of the cells which contained the irradiated centrosome stained less intensively and the nucleoli were stained mainly at the periphery (Fig. 4c).

The described difference between the nucleoli in the nuclei of irradiated and sister cells was preserved within all the time of observation (up to 24 h),



Figure 4. The ultrastructure of the nuclei of the cells 4.5 h after irradiation of the centrosome in mitosis, a, a sister cell with the non-irradiated centrosome; b, a cell with the irradiated centrosome. Bar, 3 µm.

and small dense particles in the nuclei of cells with irradiated centrosome were also preserved.

In control cells at the same stage of mitosis, a region of the cytoplasm was irradiated which was outside the spindle located away from the chromosomes at a distance equal to the length of the semispindle. Such cells passed mitosis normally and in both sister cells normal nucleoli were formed.

One of the possible reasons why the nucleoli were not restored in the cells which received the irradiated centrosome, can be the damage of the system of intracellular transport due to the absence of the radial network of micro-tubules in the interphase cells with the irradiated centrosome [1]. To verify this suggestion, the system of microtubules in the cells was damaged in a different way. Nocodazole at a concentration of 1 μ g/ml was added to the culture medium to examine the behaviour of cells in the late telophase or early interphase (20-60 min after the chromosomes began to segregate). Immunofluorescent staining by the antibodies against tubulin conducted 3-4 h after incubation in nocodazole confirmed the absence of microtubules in the cells under study (data not shown). In spite of this, the growth of the nucleoli in these cases was normal, and the small dense particles in the kaiyoplasm of the cells disappeared 2-4 h after mitosis.

DISCUSSION

Observations of nucleolus formation in the nuclei of the cells incubated in a medium with nocodazole suggest that the disturbances of nucleologenesis in the cells containing the irradiated centrosome are not related to the absence of the normal system of microtubules.

It is known that mitosis is characterized by accumulation of various proteins in the region of the centrosome, including those of nuclear origin [20]. B23 protein was localized in the mitotic centrosome by means of indirect immunocytochemistry [16]. It emerges in the prometaphase simultaneously with the degradation of the nucleolus and the nuclear envelope and is revealed in the centrosomes up to the telophase, when the restoration of the daughter cells and nucleoli begins [16]. Further on, B23 protein at the end of the telophase is localized in the cytoplasm, and in the early G_1 period it emerges in the karyoplasm. Later, in the G_1 period, simultaneously with the formation of mature nucleoli, B23 protein appears in the nucleoli and disappears in the karyoplasm [21].

Microirradiation of the centrosome does not prevent the entry of this protein into the reconstructing nucleus, but, in contrast with intact cells, its accumulation in the nucleoli is sharply slowed down or virtually blocked -the protein remains in the karyoplasm. Thus, our data indicate that the pool of B23 at the pole of the spindle is re-utilized in the subsequent interphase. Presumably, the mitotic centrosomal pool of B23 protein damaged by microirradiation in the anaphase looses its capability of being transported to the nucleolus; however, its nuclear localization is preserved.

Disturbances in the postmitotic reorganization of the nucleolus are exhibited in the slowing down of its growth, depletion of the granular component and partial inactivation of RNA synthesis [4]. The general morphology of the nuclei of the cells containing the irradiated centrosome indicates that the processes occurring in them are similar to those which are induced by the action of actinomycin D or by the injection of antibodies to RNA polymerase I, namely: segregation of the nucleolar components, *sharp* depletion of the granules and B23, abnormally long presence of the small dense particles described above [12]. Since the irradiation of the region of the mitotic spindle fails to lead to any noticeable changes in the course of nucleologenesis, it could be suggested that during the mitosis a factor(s) which directly affect(s) the assembly of the nucleoli are located in the centrosome.

One of the causes of abnormal nucleologenesis can be the damage of the centrosomal pool of B23, which is somehow involved in the restoration of the nucleoli during the mitosis, by irradiation. The data that, besides the regulation of the ribosome assembly, B23 can activate the DNA polymerase

activity *in vitro* [22] are indirect evidence that this protein can play the role the an rDNA transcription activator. The fact that the irradiation of the centro-some with UV light decreases the level of transcription in the early interphase approximately twofold as compared with sister cells is also in favour of the existence of nuclear protein in the spindle pole in the anaphase [4].

Migration of B23 protein to the reconstructing nucleus occurs simultaneously in irradiated and sister cells as well as the formation of small dense particles in the karyoplasm. In a cell with the irradiated centrosome, small dense particles are then preserved for many hours, whereas in a sister cell they disappear in 2-4 h. This implies that the material of small dense particles, most probably, does not include mitotic centrosomal B23 protein. Presumably, dense particles are formed due to the perichromosome pool of B23, which is transferred into the daughter cells on the surface of the chromosome [19, 21].

Thus, microirradiation of the centrosome in the anaphase leads to the disturbance of the formation of normal nucleoli after the transition of the cells into the interphase, which can be due to the damage of B23 protein, accumulated during the mitosis in the region of the spindle pole.

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